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1,25-Dihydroxyvitamin D₃ is a negative endocrine regulator of the renin-angiotensin system

See related Commentary on pages 155–156.

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Inappropriate activation of the renin-angiotensin system, which plays a central role in the regulation of blood pressure, electrolyte, and volume homeostasis, may represent a major risk factor for hypertension, heart attack, and stroke. Mounting evidence from clinical studies has demonstrated an inverse relationship between circulating vitamin D levels and the blood pressure and/or plasma renin activity, but the mechanism is not understood. We show here that renin expression and plasma angiotensin II production were increased severalfold in vitamin D receptor–null (VDR-null) mice, leading to hypertension, cardiac hypertrophy, and increased water intake. However, the salt- and volume-sensing mechanisms that control renin synthesis are still intact in the mutant mice. In wild-type mice, inhibition of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] synthesis also led to an increase in renin expression, whereas 1,25(OH)₂D₃ injection led to renin suppression. We found that vitamin D regulation of renin expression was independent of calcium metabolism and that 1,25(OH)₂D₃ markedly suppressed renin transcription by a VDR-mediated mechanism in cell cultures. Hence, 1,25(OH)₂D₃ is a novel negative endocrine regulator of the renin-angiotensin system. Its apparent critical role in electrolytes, volume, and blood pressure homeostasis suggests that vitamin D analogues could help prevent or ameliorate hypertension.

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Introduction

The renin-angiotensin system is a regulatory cascade that plays an essential role in the regulation of blood pressure, electrolyte, and volume homeostasis. The first and rate-limiting component of this cascade is renin, a protease synthesized and secreted predominantly by the juxtaglomerular (JG) apparatus in the nephron. Renin cleaves angiotensin I (Ang I) from liver-derived angiotensinogen, which is then converted to Ang II by the angiotensin-converting enzyme. Ang II, through binding to its receptors, exerts diverse actions that affect the electrolyte, volume, and blood pressure homeostasis (1). Inappropriate stimulation of the renin-angiotensin system has been associated with hypertension, heart attack, and stroke.

The renin-producing granulated cells are mainly located in the afferent glomerular arterioles in the kidney (2). It is well established that renin secretion is regulated by renal perfusion pressure, renal sympar-

thetic nerve activity, and tubular sodium load (1, 2). Renin secretion is stimulated by factors such as prostaglandins, NO, and adrenomedullin, and inhibited by other factors, including Ang II (feedback), endothelin, vasopressin, and adenosine (1, 2). Stimulation of renin secretion is often mediated by an increase in intracellular cAMP and is accompanied by increases in renin gene transcription (3). In the renin gene promoter, several cAMP response elements have been identified. Recently, steroid hormone receptors LXR α and RAR/RXR complex, transcriptional factors CREB/CREM and USF1/USF2, and HOX gene family members have been found to be involved in the activation of murine renin gene transcription (4–7).

Vitamin D is a primary regulator of calcium homeostasis. Genetic inactivation of either the vitamin D receptor (VDR), a member of the nuclear receptor superfamily that mediates the action of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], or 25-hydroxyvitamin D₃ 1 α -hydroxylase, the rate-limiting enzyme for the biosynthesis of 1,25(OH)₂D₃, results in impaired calcium homeostasis, leading to hypocalcemia, secondary hyperparathyroidism, and rickets (8–11). However, the wide tissue distribution of VDR suggests that the vitamin D endocrine system has additional physiological functions beyond calcium homeostasis. Indeed, vitamin D and VDR have been shown to play important roles in the immune system, cardiovascular system, reproductive system, and hair growth.

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Nonstandard abbreviations used: vitamin D receptor–null (VDR-null); 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]; juxtaglomerular (JG); angiotensin I (Ang I); serum intact parathyroid hormone (iPTH); cyclooxygenase-2 (COX-2).

In the last two decades, clinical studies have revealed an inverse relationship between the plasma $1,25(\text{OH})_2\text{D}_3$ concentration and the blood pressure and/or the plasma renin activity in both normotensive men and patients with essential hypertension (12–16). Ultraviolet light exposure, which is required for vitamin D biosynthesis, is inversely related to the rise of blood pressure and the prevalence of hypertension in the general population and was shown to have blood pressure-lowering effects (17, 18). Furthermore, it has been reported that vitamin D_3 supplementation reduces blood pressure in patients with essential hypertension (19, 20), and $1,25(\text{OH})_2\text{D}_3$ treatment reduces blood pressure, plasma renin activity, and Ang II levels in hyperparathyroidism patients (21, 22). Despite the significance of these observations, however, the mechanism underlying the relationship between vitamin D and blood pressure and plasma renin activity is unknown.

To explain these observations, we hypothesized that vitamin D is a negative regulator of renin expression *in vivo*. If this hypothesis is correct, disruption of the vitamin D signaling pathway should lead to a deregulated elevation of renin expression, and an increase in serum vitamin D levels should lead to a suppression of renin expression. We have tested this hypothesis. Here we provide *in vivo* and *in vitro* evidence that establishes vitamin D as a potent negative endocrine regulator of the renin-angiotensin system.

Methods

Animals and treatment. The generation and characterization of $\text{VDR}^{-/-}$ and $\text{Gcm2}^{-/-}$ mice have been described previously (10, 23). $\text{VDR}^{-/-}$ and $\text{Gcm2}^{-/-}$ mice were generated through breeding of heterozygous mice and identified by PCR with tail genomic DNA as the template, and the wild-type littermates were used as controls in all experiments. Mice were housed in a pathogen-free barrier facility in a 12-hour light/12-hour dark cycle, and fed an autoclaved standard rodent chow. To normalize the blood ionized calcium level of $\text{VDR}^{-/-}$ mice, 2-month-old animals were placed on the HCa-Lac diet (Harlan Teklad, Madison, Wisconsin, USA) containing 2% calcium, 1.25% phosphorus, 4 IU/g vitamin D, and 20% lactose (24) for 5 weeks. To increase the sodium load, mice were fed the normal rodent diet supplemented with 8% NaCl for 1, 3, 5, and 7 days. In dehydration experiments, mice were restricted from water, but had free access to food, for 24 hours before sacrifice. To block $1,25(\text{OH})_2\text{D}_3$ synthesis, 1.5-month old wild-type mice were placed on the normal diet supplemented with 2.5% strontium chloride until hypocalcemia was detected. To investigate the effect of $1,25(\text{OH})_2\text{D}_3$ *in vivo*, wild-type mice were injected intraperitoneally with vehicle or 30 pmol of $1,25(\text{OH})_2\text{D}_3$ dissolved in propylene glycol as detailed in Results. In all experiments, mice were sacrificed by exsanguination under anesthesia, and the blood was collected into ice-cold tubes for serum isolation, or into

ice-cold tubes containing 50 μl of EDTA (pH 8.0) and 100 U/ml aprotinin for plasma isolation. The determination of water and food intake, as well as urine collection, was carried out by using metabolic cages.

Measurement of blood and urine parameters. The concentration of blood ionized calcium was determined using a 634 Ca^{++}/pH analyzer (Chiron Diagnostics, East Walpole, Massachusetts, USA) from 50 μl of whole blood obtained from tail snipping. Blood glucose concentrations were determined by using One Touch SureStep test strips (LifeScan Inc., Milpitas, California, USA). Serum intact parathyroid hormone (iPTH) was determined using a commercial ELISA kit (Immutopics Inc., San Clemente, California, USA). The concentration of serum and urinary Na^+ , K^+ , and creatinine was determined by a CX5 Autoanalyzer (Beckman Coulter Inc., Brea, California, USA) as described previously (25).

Measurement of Ang II. Mouse plasma Ang II concentrations were determined by RIA, using a commercial RIA kit (Phoenix Pharmaceuticals Inc., Mountain View, California, USA) according to the manufacturer's instructions.

Measurement of blood pressure. Mouse blood pressure was determined as described previously (26). Briefly, mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The left carotid artery was isolated from surrounding tissues, and cannulated with a polyethylene catheter filled with sterile PBS containing heparin (50 U/ml) under a dissecting microscope. Arterial blood pressure was measured using a pressure transducer model 60-3002 (Harvard Apparatus Co., Hilliston, Massachusetts, USA) and recorded. To investigate whether the increase in blood pressure in $\text{VDR}^{-/-}$ mice was directly due to the increase in the Ang II level, wild-type and $\text{VDR}^{-/-}$ mice were treated with captopril (100 mg/d/kg body weight, dissolved in drinking water) for 5 days before blood pressure was determined. Wild-type and $\text{VDR}^{-/-}$ mice fed normal drinking water were used as controls.

Immunohistochemistry. Kidneys freshly dissected from wild-type and $\text{VDR}^{-/-}$ mice were fixed overnight with 4% formaldehyde in PBS (pH 7.2), processed, embedded in paraffin, and cut into 5- μm sections with a Leica Microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The slides were stained with a rabbit polyclonal anti-renin antiserum (1:1,600 dilution) (kindly provided by T. Inagami, Vanderbilt University, Nashville, Tennessee, USA). After incubation with a peroxidase-conjugated anti-rabbit IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, USA), the renin signal was visualized with a DAB peroxidase substrate kit (Vector Laboratories Inc., Burlingame, California, USA), followed by a light hematoxylin counterstaining.

RNA isolation and Northern blot. The kidney and liver were dissected and immediately placed into Trizol reagent (Invitrogen Life Technologies, Carlsbad, California, USA) for total RNA isolation according to the manufacturer's instruction. To determine renin or

angiotensinogen mRNA expression, total RNA (20 µg/lane) was separated on a 1.2% agarose gel containing 0.6 M formaldehyde, transferred onto a nylon transfer membrane (Micron Separations Inc., Westborough, Massachusetts, USA), and cross-linked in an ultraviolet cross-linker (Bio-Rad Laboratories Inc., Hercules, California, USA). Hybridization was performed as described previously (25). Mouse renin and angiotensinogen cDNA probes were labeled with ^{32}P -dATP (ICN Biomedicals Inc., Costa Mesa, California, USA) using the Prime-a-Gene Labeling System (Promega Corp., Madison, Wisconsin, USA). After hybridization and washing, membranes were exposed to x-ray films at -80°C for autoradiography. The relative amount of mRNA was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, California, USA) and normalized with 36B4 mRNA as described previously (25).

As4.1 cell culture and transfection. As4.1 cells (American Type Culture Collection, Manassas, Virginia, USA) were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO_2 . For transient transfection, the cells were grown in 10-cm dishes to 50% confluence and transfected with pcDNA3.1, pcDNA-hVDR, or pcDNA-PTH/PTHrPR plasmid (10 µg DNA per dish) by the standard calcium phosphate method. Twenty-four hours after transfection, the cells were treated for 24 hours with 5×10^{-8} M $1,25(\text{OH})_2\text{D}_3$ or ethanol in serum-free media, or with different doses of bovine PTH(1-34) as indicated. Total RNA was isolated and analyzed for renin mRNA expression by Northern blot. For stable transfection, As4.1 cells were transfected with pcDNA3.1 or pcDNA-hVDR plasmid by the use of SuperFect reagent (QIAGEN Inc., Valencia, California, USA) and selected with 350 µg/ml of G418 for 2 weeks. Individual colonies were picked, expanded, and selected for VDR expression. The As4.1-hVDR stable clones were treated with different doses of $1,25(\text{OH})_2\text{D}_3$ for 24 hours in serum-free media, and total RNA was analyzed by Northern blot to examine renin expression.

Renin gene promoter analysis. Plasmid pR1C-4.1CAT that contains 4.1 kb 5'-flanking sequence of mouse *Ren-1*^c gene (27) was provided by K.W. Gross (Roswell Park Cancer Institute, Buffalo, New York, USA). To generate pGL-117bp reporter plasmid, the 123-bp renin minimal promoter fragment (+6 to -117) was released from pR1C-4.1CAT with XbaI and BamHI and inserted into the HindIII site of pGL3-basic vector (Promega Corp.). To generate pGL-4.1kb reporter plasmid, the BamHI fragment (-4.1 kb to -118 bp) from pR1C-4.1CAT was inserted into the BglII site of pGL-117bp. To analyze the activity of renin gene promoter, As4.1-hVDR cells were transfected with the reporter plasmids by electroporation according to the method of Shi et al. (5) using a Gene Pulser (Bio-Rad Laboratories Inc.). pCMV-β-galactosidase (β-gal) plasmid was cotransfected as an internal control. pGL3-control plasmid (Promega Corp.) was used as the positive control. The transfected cells were treated with

ethanol or 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ in Opti-MEM medium (Invitrogen Life Technologies) containing 2% charcoal-treated FBS 4 hours after electroporation, and luciferase activity was determined at 48 hours after initial transfection using the Luciferase Assay System (Promega Corp.). Luciferase activity was normalized to β-gal activity obtained from the same electroporation, and presented as fold induction based on the basal activity of pGL3-basic empty vector determined in the same experiment.

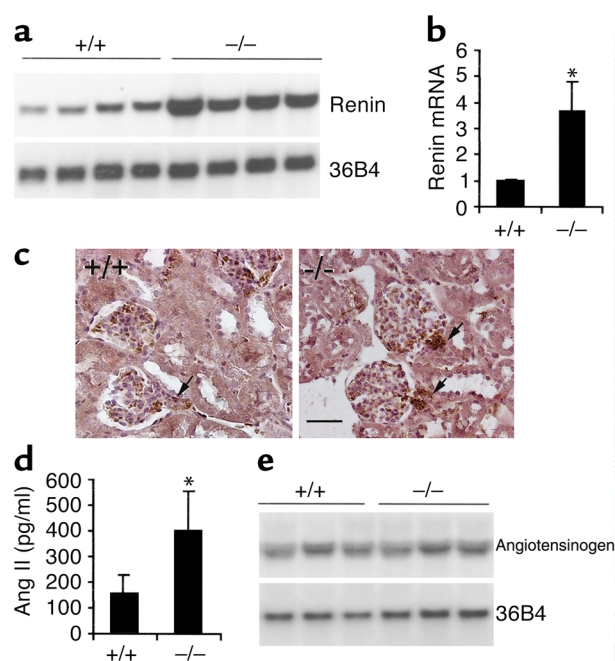
Statistical analysis. Data were presented as mean ± SD and analyzed with Student's *t* test to assess significance. *P* values of 0.05 or lower were considered statistically significant.

Results

Renin expression and plasma Ang II production are elevated in VDR-null mice. To test our hypothesis we first analyzed VDR^{-/-} mice. We reasoned that if the hypothesis is correct, renin expression should be increased in the mutant mice because of the disruption of the vitamin D signaling pathway. By quantitative Northern blot analysis, we found that the renin mRNA level in the kidneys of adult VDR^{-/-} mice was more than threefold higher than that of wild-type littermates (Figure 1, a and b). Immunohistochemical analysis of the renal cortex with an anti-renin antibody confirmed a dramatic increase in renin immunoreactivity in the afferent glomerular arterioles of the JG region in VDR^{-/-} mice (Figure 1c). The plasma Ang II level of VDR^{-/-} mice was also increased more than 2.5-fold as compared with that of wild-type mice (Figure 1d). However, the expression of angiotensinogen, the precursor of Ang II, in the liver of VDR^{-/-} mice was the same as in wild-type mice (Figure 1e), suggesting that the increase in plasma Ang II was mainly due to the increase in renin activity.

VDR-null mice are hypertensive. Ang II is a potent vasoconstrictor (1). We therefore compared the blood pressure of VDR^{-/-} and wild-type mice. We found that both the systolic and the diastolic pressures of VDR^{-/-} mice were significantly higher (>20 mmHg) than those of wild-type littermates (Figure 2a), indicating that VDR^{-/-} mice are hypertensive. Furthermore, the heart weight/body weight ratios of the mutant mice were also significantly higher (Figure 2b), suggesting that the adult VDR^{-/-} mice had developed cardiac hypertrophy. When the mice were treated with captopril, an angiotensin-converting enzyme inhibitor, the blood pressure of both wild-type and VDR^{-/-} mice was reduced as expected. However, no difference was seen between the blood pressures of the treated wild-type and VDR^{-/-} mice (Figure 2c). This result confirms that the increase in the blood pressure of VDR^{-/-} mice is due to renin and plasma Ang II elevation.

VDR-null mice show abnormal drinking behavior. Ang II is known to be a very potent stimulus for thirst and salt craving as well as an inducer of intestinal water and sodium absorption (1, 28). We therefore measured water and food intake as well as blood and

**Figure 1**

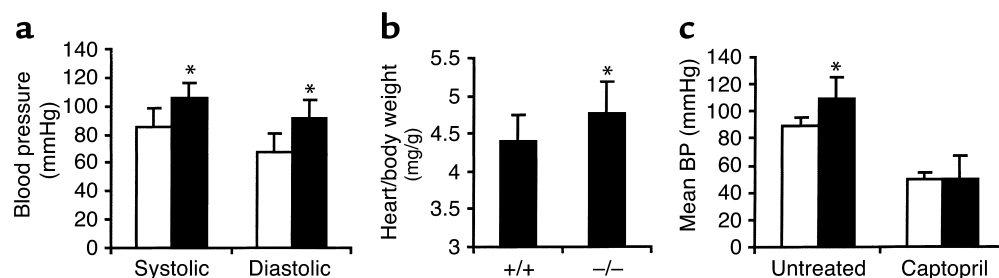
Effect of VDR inactivation on renin expression and plasma Ang II production. (a) Renin mRNA expression in the kidney. Kidney total RNAs were isolated from wild-type (+/+) and VDR^{-/-} (-/-) mice and analyzed by Northern blot. The same membrane was sequentially hybridized with mouse renin and 36B4 cDNA probes. Each lane represents an individual animal. (b) Quantitative results of the Northern blot analyses shown in a. Values represent the ratio of renin mRNA to 36B4 mRNA. **P* < 0.001 vs. wild-type mice. (c) Immunohistochemical staining of the kidney cortex from wild-type and VDR^{-/-} mice with anti-renin antiserum. Arrows indicate the afferent glomerular arterioles in the JG region. Scale bar, 25 μ m. (d) Plasma Ang II concentrations in wild-type and VDR^{-/-} mice. **P* < 0.001 vs. wild-type mice; *n* = 15 in each group. (e) Liver angiotensinogen mRNA expression in wild-type and VDR^{-/-} mice, determined by Northern blot. The membrane was sequentially hybridized with mouse angiotensinogen and 36B4 cDNA probes. Each lane represents an individual mouse.

urinary electrolyte parameters. As shown in Table 1, VDR^{-/-} mice ingested about twice as much water as the wild-type littermates and, consequently, excreted approximately twice as much urine. The abnormal drinking behavior is not due to diabetes, since the blood glucose and insulin levels of VDR^{-/-} mice were normal (Table 2). Food intake of VDR^{-/-} mice was similar to that of wild-type mice, but VDR^{-/-} mice excreted 37% and 19% more Na⁺ and K⁺ in the urine, respectively (Table 1), while maintaining a normal concentration of blood Na⁺ and K⁺ (Table 2). Thus, VDR^{-/-} mice appeared to have an increase in intestinal salt absorption due to the Ang II elevation.

VDR-null mice respond properly to salt load or volume change. As renin production is very sensitive to changes in tubular salt load or extracellular fluid volume (29, 30), we investigated the effect of high-salt diet or dehydration on the expression of renin in VDR^{-/-} and wild-type littermates. When placed on a normal diet supplemented with 8% NaCl, both VDR^{-/-} and wild-type

mice responded by reducing the expression of renin mRNA, but VDR^{-/-} mice still maintained a significantly higher renin mRNA level even after 7 days on the high-salt diet (Figure 3a). Similar changes were seen in the plasma Ang II levels in these animals (Figure 3b). On the other hand, when the mice were dehydrated for 24 hours, which leads to hypovolemia, they responded by increasing renin mRNA synthesis, but the increase in wild-type mice was more dramatic than in VDR^{-/-} mice (Figure 3c), suggesting that the basal renin production in VDR^{-/-} mice was already near the maximal capacity. The changes of plasma Ang II concentrations in the dehydrated mice were consistent with the changes in the renin expression (Figure 3d). These observations indicated that, despite a high basal renin synthesis, the regulatory mechanisms activated by tubular salt load changes or volume depletion are still intact in VDR^{-/-} mice. These data also suggest that the elevation of the basal renin expression in VDR^{-/-} mice is through a mechanism different from that of the physiological inducers.

Inhibition of 1,25(OH)₂D₃ biosynthesis also leads to renin upregulation. Dietary strontium has been shown to block the biosynthesis of 1,25(OH)₂D₃ and has been widely used to render animals vitamin D-deficient (31). To confirm that the disruption of the vitamin D

**Figure 2**

Effect of VDR inactivation on blood pressure and heart weight/body weight ratio. (a) Systolic and diastolic blood pressures of wild-type (white bars) and VDR^{-/-} (black bars) mice. **P* < 0.01 vs. corresponding wild-type mice; *n* = 9 for wild-type mice; *n* = 8 for VDR^{-/-} mice. (b) Ratio of heart weight to body weight of wild-type and VDR^{-/-} mice. **P* < 0.05 vs. wild-type mice; *n* = 9 in each genotype. (c) Mean blood pressure (BP) of wild-type (white bars) and VDR^{-/-} (black bars) mice untreated or treated with captopril for 5 days. **P* < 0.05 vs. corresponding untreated wild-type mice; *n* = 4 in each genotype in each group.

Table 1

Twenty-four-hour water and food intake, urinary volume, and urinary electrolyte concentrations

	Wild-type	VDR ^{-/-}	P value
Water (ml/mouse/day)	2.7 ± 0.3 (n = 29)	5.4 ± 0.4 (n = 29)	< 0.01
Food (g/kg BW/day)	138.9 ± 12.2 (n = 29)	142.9 ± 13.1 (n = 29)	NS
Urine (ml/mouse/day)	1.1 ± 0.4 (n = 9)	1.8 ± 0.5 (n = 9)	< 0.01
Urinary Na ⁺ /Cr	2.9 ± 0.6 (n = 9)	3.9 ± 0.7 (n = 9)	0.09
Urinary K ⁺ /Cr	3.8 ± 0.4 (n = 9)	4.5 ± 0.4 (n = 9)	< 0.01

BW, body weight; Cr, creatinine; NS, not significant.

signaling can lead to renin upregulation, we treated wild-type mice with strontium. We monitored the blood ionized calcium, instead of the blood 1,25(OH)₂D₃ level, during the treatment because of the extreme difficulty of measuring the serum 1,25(OH)₂D₃ concentration in live mice. As shown in Figure 4, after 7 weeks of treatment, the wild-type mice became hypocalcemic (Figure 4c), indicating that the concentration of 1,25(OH)₂D₃ was already reduced, since 1,25(OH)₂D₃ is required to maintain the calcium homeostasis. As expected, the treated mice showed a significant increase in renin mRNA expression (Figure 4, a and b), consistent with the suppressive role of 1,25(OH)₂D₃ in renin expression.

1,25(OH)₂D₃ treatment suppresses renin expression in wild-type mice. To further confirm that 1,25(OH)₂D₃ indeed suppresses renin expression in vivo, we treated wild-type mice with 1,25(OH)₂D₃ or vehicle and then determined the renin mRNA level in the kidney. After two doses of 1,25(OH)₂D₃ (30 pmol/dose) in 2 consecutive days, renal renin expression was decreased by 35%, and after five doses in 3 days, the expression was decreased by 50% (Figure 5, a and b). As a control, the mRNA of renal calbindin-D9k, a well-known vitamin D target gene (25), was significantly increased by the 1,25(OH)₂D₃ treatment (Figure 5a). Thus, the data obtained from VDR^{-/-} mutant mice and from strontium- and 1,25(OH)₂D₃-treated wild-type mice confirm the existence of a negative regulatory interaction between vitamin D and the renin-angiotensin system in vivo.

Elevation of renin expression is independent of hypocalcemia. As vitamin D is a primary regulator of calcium homeostasis, changes in the vitamin D status inevitably alter the blood levels of calcium and PTH in animals. For instance, adult VDR^{-/-} mice developed hypocalcemia and secondary hyperparathyroidism (10). As shown in Figure 6, their blood ionized calcium level was decreased by 30% and serum PTH concentration increased about 150-fold at 3 months of age (Figure 6, a and b). A key question, therefore, is whether the effect of VDR inactivation on renin expression in vivo is direct, or is only secondary to changes in the blood calcium or PTH level, as hypocalcemia may reduce the intracellular calcium concentration and cause the renin upregulation (32), and high PTH may also stimulate renin secretion (33). To

address this question, we examined 20-day-old VDR^{-/-} mice that were still normocalcemic (Figure 6a) but already showed a six-fold increase in the serum PTH level (Figure 6b), likely due to the lack of the VDR-mediated vitamin D inhibition of PTH biosynthesis (34). A significant increase in renin expression was seen in these preweaned VDR^{-/-} mice (Figure 6, c and d). We also examined the adult VDR^{-/-} mice treated with the HCa-Lac diet containing 2% calcium, 1.25% phosphorus, and 20% lactose (24). Five weeks of dietary treatment normalized the blood ionized calcium level in VDR^{-/-} mice (Figure 6a) and reduced the serum PTH concentration of VDR^{-/-} mice to about seven times the wild-type value (Figure 6b) but had no effects on the concentration of blood electrolytes (Table 2). However, renin mRNA and plasma Ang II levels in these normocalcemic adult VDR^{-/-} mice were still significantly elevated (Figure 6, e–g). Similarly, their water intake and urinary excretion were also significantly higher (data not shown). In addition, renin expression was still elevated in VDR^{-/-} mice whose alopecia was rescued by targeted expression of human VDR in the skin (35), indicating that the upregulation of renin expression is not due to alopecia (data not shown).

To further exclude the possibility that hypocalcemia may increase renin expression, we examined renin expression in Gcm2^{-/-} mice, which lack the parathyroid glands (Gcm2 is a master regulatory gene for parathyroid gland development) but have normal circulating PTH (derived from the thymus) and 1,25(OH)₂D₃ concentrations (23). Although the blood ionized calcium of Gcm2^{-/-} mice was as low as that of VDR^{-/-} mice, no increase in renin mRNA expression was detected in Gcm2^{-/-} mice (data not shown). Taken together, these data demonstrate that the elevation of renin expression is not due to hypocalcemia but resulted from VDR inactivation per se and/or hyperparathyroidism.

Vitamin D directly suppresses renin expression in cell cultures. To prove that vitamin D directly suppresses

Table 2

Blood parameters under normal and high-calcium dietary conditions

	Wild-type	VDR ^{-/-}	P value
Normal diet			
Na ⁺ (mmol/l)	148.7 ± 4.9 (n = 10)	148.3 ± 2.6 (n = 8)	NS
K ⁺ (mmol/l)	5.2 ± 0.9 (n = 10)	4.8 ± 0.6 (n = 8)	NS
Creatinine (mg/dl)	0.29 ± 0.1 (n = 10)	0.24 ± 0.1 (n = 8)	NS
Glucose (mg/dl)	116.2 ± 4.1 (n = 3)	115 ± 13.3 (n = 5)	NS
Insulin (ng/ml)	0.42 ± 0.2 (n = 6)	0.38 ± 0.1 (n = 8)	NS
HCa-Lac diet for 5 weeks			
Na ⁺ (mmol/l)	143.7 ± 3.9 (n = 5)	143.1 ± 1.7 (n = 5)	NS
K ⁺ (mmol/l)	4.4 ± 0.9 (n = 5)	4.2 ± 0.3 (n = 5)	NS
Creatinine (mg/dl)	0.26 ± 0.1 (n = 5)	0.25 ± 0.1 (n = 5)	NS

NS, not significant.

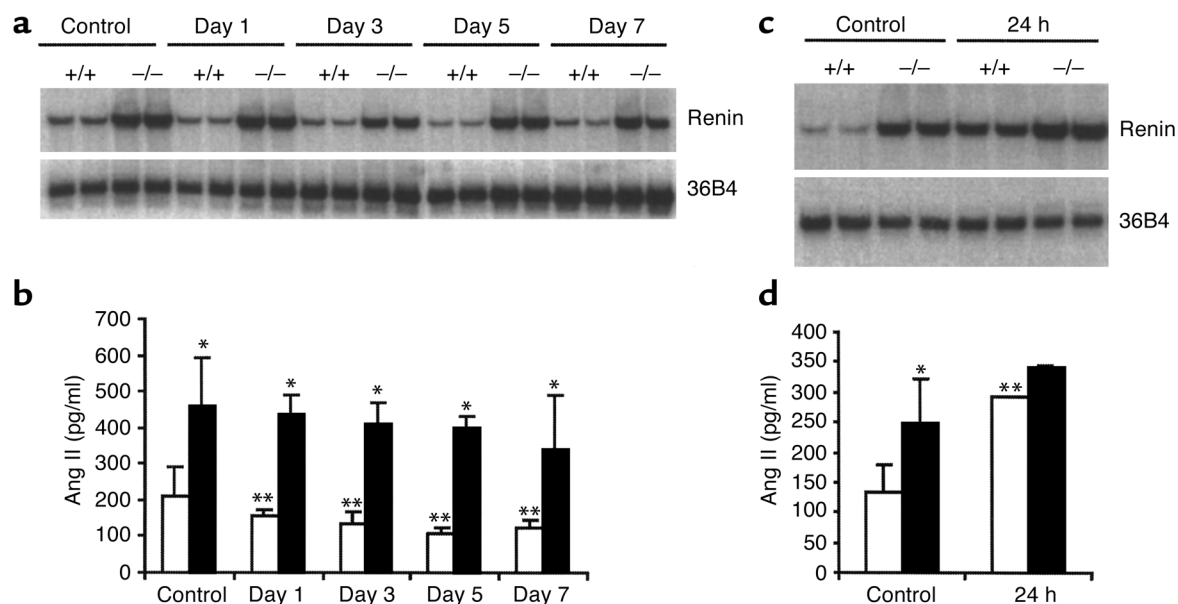


Figure 3

Effect of high sodium load and volume depletion on renin mRNA expression and plasma Ang II production in wild-type and VDR^{-/-} mice. (a) Northern blot analysis of renal renin mRNA from mice treated, for different numbers of days as indicated, with the normal rodent diet supplemented with 8% NaCl. Each lane represents an individual mouse. Control mice were untreated. (b) Plasma Ang II concentrations in the 8% NaCl diet-treated animals. White bars, wild-type mice; black bars, VDR^{-/-} mice. *P < 0.01 vs. corresponding wild-type mice at the same time point; **P < 0.05 vs. untreated control wild-type mice; n = 3 in each genotype at each time point. (c) Northern blot analysis of renal renin mRNA expression in mice dehydrated for 24 hours (24 h). Each lane represents an individual mouse. Control mice were untreated. (d) Plasma Ang II levels in untreated control and dehydrated (24 h) mice. White bars, wild-type mice; black bars, VDR^{-/-} mice. *P < 0.01 vs. corresponding wild-type mice; **P < 0.01 vs. untreated control wild-type mice; n = 3 in each genotype in each group.

renin gene expression, we examined the effect of 1,25(OH)₂D₃ treatment on renin mRNA expression in As4.1 cells, a JG cell-like cell line that was derived from kidney tumors of SV40 T antigen transgenic mice and maintains a high level of renin synthesis (36). Treatment with 5 × 10⁻⁸ M 1,25(OH)₂D₃ caused a moderate reduction in renin mRNA expression; however, when the cells were transiently transfected with the pcDNA-hVDR plasmid that contained the full-length coding sequence of human VDR cDNA, the same 1,25(OH)₂D₃ treatment reduced renin mRNA expression by about 90% (Figure 7, a and b). Thus, 1,25(OH)₂D₃ directly suppresses renin expression in a VDR-dependent manner.

To investigate whether the high PTH level in VDR^{-/-} mice also contributes to the renin upregulation, As4.1 cells were treated with different doses of PTH(1-34), or transfected with the plasmid pcDNA-PTH/PTHrPR that contains the full-length rat PTH/PTHrP receptor

cDNA and then treated with PTH(1-34). No increase in renin expression was observed in either of the PTH-treated cells (Figure 7c).

Vitamin D suppresses renin gene promoter activity. It has been shown that As4.1 cells have lost expression of some nuclear receptors, such as LXR (4), and we found that VDR mRNA transcript was undetectable in As4.1 cells by Northern blot (not shown). To confirm the VDR-mediated suppression of renin expression, As4.1 clones stably transfected with the pcDNA3.1 vector or

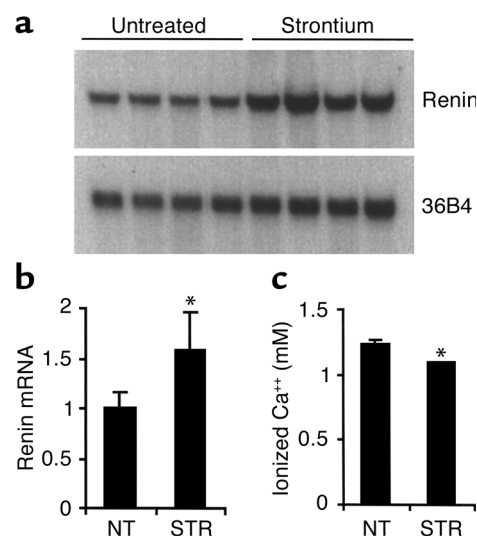
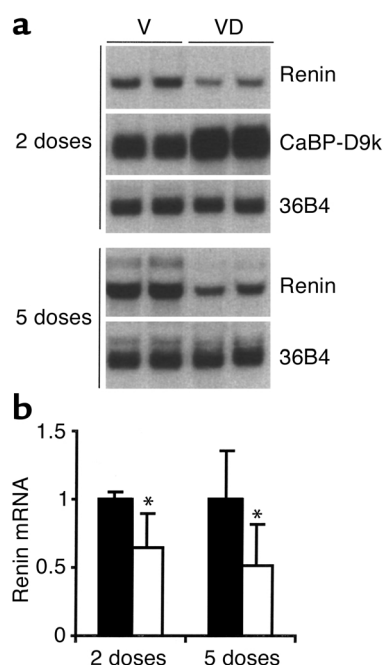


Figure 4

Elevation of renin expression in strontium-treated wild-type mice. Two-month-old wild-type mice were fed the normal diet supplemented with 2.5% strontium chloride for 7 weeks before sacrifice. (a) Northern blot analysis of renin mRNA expression in the kidney from untreated and strontium-treated wild-type mice. Each lane represents an individual animal. (b) Quantitative results of the Northern analysis. (c) Blood ionized calcium concentration determined at the end of the treatment (n = 5 in each group). *P < 0.01 vs. untreated value (in b and c). NT, not treated; STR, strontium-treated.

**Figure 5**

1,25-Dihydroxyvitamin D₃ suppresses renin expression in wild-type mice. (a) Wild-type mice (3 months old) were injected intraperitoneally with two or five doses of 30 pmol of 1,25(OH)₂D₃ (VD) dissolved in propylene glycol or vehicle (V). The two doses were given in 2 consecutive days at 9 am. The five doses were given in 3 consecutive days at 9 am and 7 pm on the first 2 days and 9 am on the third day. Total renal RNA was isolated 6 hours after the last injection. Renin, calbindin-D9k (CaBP-D9k), and 36B4 mRNA levels were determined by Northern blot analysis. (b) Quantitation of renin mRNA levels. Black bars, vehicle treatment; white bars, 1,25(OH)₂D₃ treatment. **P* < 0.05 vs. vehicle treatment; *n* ≥ 3 in each group.

pcDNA-hVDR were established (Figure 8a). When the stable clones were treated with 1,25(OH)₂D₃, a dose-dependent suppression of renin expression was seen in As4.1-hVDR cells, but not in As4.1-pcDNA cells (Figure 8b). Again, the level of renin mRNA was reduced by about 90% in As4.1-hVDR cells treated with 10⁻⁸ M 1,25(OH)₂D₃. Time-course studies showed that the suppression of renin mRNA was evident after 6 hours of 1,25(OH)₂D₃ treatment (data not shown). To investigate whether the VDR-mediated suppression is at the transcriptional level, we measured the activity of the renin gene promoter in As 4.1-hVDR cells. As shown in Figure 8c, transfection of the cells with the pGL-4.1kb

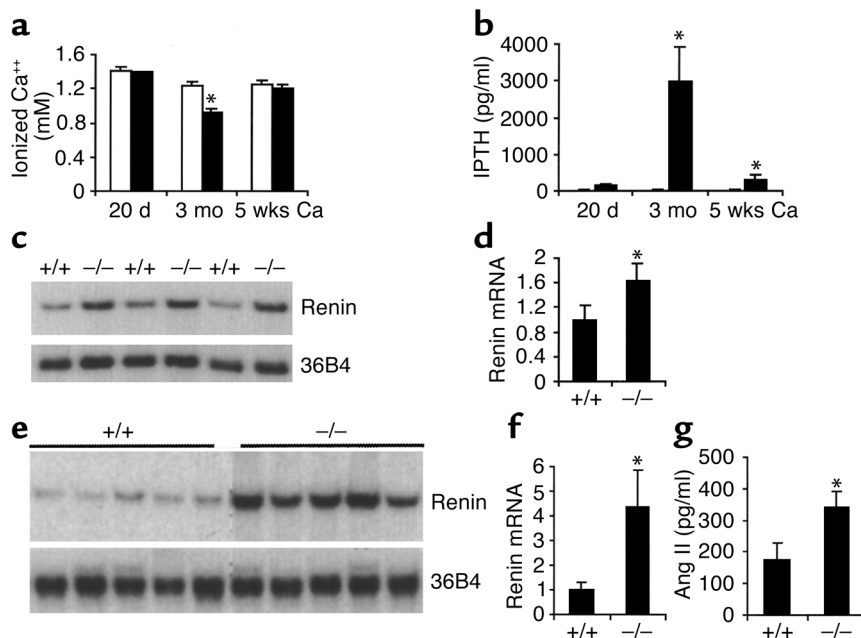
reporter plasmid containing the 4.1-kb 5'-flanking sequence of the murine renin gene (*Ren-1*) (27) resulted in a 25-fold increase in luciferase activity. Treatment of the transfected cells with 1,25(OH)₂D₃ reduced the activity of the 4.1-kb renin gene promoter by more than 80% but had no effect on the activity of the SV40 promoter in pGL3-control plasmid. Thus, the suppression of the renin gene promoter by 1,25(OH)₂D₃ is potent and specific. As reported previously, the 117-bp 5'-flanking fragment had very low activity (27). These results provide compelling evidence that 1,25(OH)₂D₃ directly and negatively regulates renin gene transcription through a VDR-mediated mechanism.

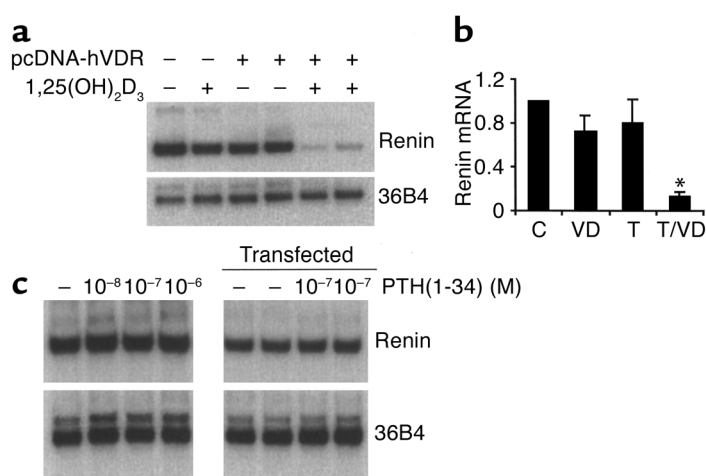
Discussion

The primary physiological function of the renin-angiotensin system is to maintain vascular resistance and extracellular fluid volume homeostasis. This is mainly accomplished by the regulatory actions of Ang II on the peripheral vasculature, heart, CNS, kidney, and adrenal glands. As the rate-limiting component of the renin-angiotensin cascade, renin secretion and production is mostly stimulated by volume or salt depletion, reduction in renal vascular perfusion pres-

Figure 6

Renin upregulation is independent of the calcium status. (a and b) Blood ionized calcium levels (a) and serum iPTH concentrations (b) in wild-type (white bars) and VDR^{-/-} (black bars) mice at 20 days of age, at 3 months of age, or treated with the HCa-Lac diet for 5 weeks (5 wks Ca). **P* < 0.01 vs. corresponding wild-type value; *n* ≥ 5 in each group. Note that the white bars are barely visible in b. (c) Northern blot analysis of renal renin mRNA from 20-day-old wild-type and VDR^{-/-} mice. (d) Quantitative data of the Northern blot analyses from 20-day-old mice. **P* < 0.001 vs. wild-type mice. (e) Northern blot analysis of renin mRNA expression in the kidney of wild-type and VDR^{-/-} mice treated with the HCa-Lac diet for 5 weeks. (f) Quantitative result of the Northern blot analysis in shown in e. **P* < 0.001 vs. wild-type mice. (g) Plasma Ang II concentrations of the mice analyzed in e. **P* < 0.01 vs. wild-type mice; *n* = 5 in each genotype.



**Figure 7**

Suppression of renin mRNA expression by 1,25(OH)₂D₃ in As4.1 cells. **(a)** As4.1 cells were transiently transfected with (+) or without (-) pcDNA-hVDR plasmid containing the full-length human VDR cDNA and then treated with 5×10^{-8} M 1,25(OH)₂D₃ (+) or ethanol (-) for 24 hours. Total cellular RNA was isolated and analyzed by Northern blot with renin and 36B4 cDNA probes. **(b)** Quantitative results of Northern blot analyses obtained from three independent experiments. C, ethanol-treated; VD, 1,25(OH)₂D₃-treated; T, transfected with pcDNA-hVDR and treated with ethanol; T/VD, transfected with pcDNA-hVDR and treated with 1,25(OH)₂D₃. * $P < 0.001$ vs. C, VD, or T value. **(c)** Renin mRNA expression in As4.1 cells (left panel), and in As4.1 cells transfected with pcDNA-PTH/PTHrP containing the rat PTH/PTHrP receptor cDNA (right panel) and treated with bovine PTH(1-34) as indicated. -, untreated.

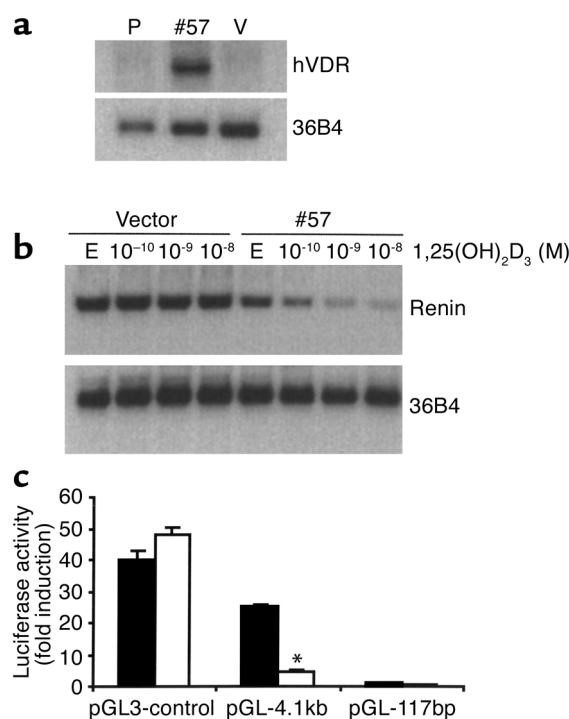
sure, and sympathetic nerve activity (1, 2). In the present study we demonstrate that VDR-null mice have a sustained elevation of renin expression while still maintaining a normal level of blood electrolytes. The augmentation of renin synthesis leads to increased plasma Ang II production from angiotensinogen, which drives VDR-null mice to increase water intake and intestinal salt absorption, since Ang II is a very potent thirst-inducing agent that acts on the CNS, as well as a potent stimulator of intestinal sodium absorption (1, 28). As a result, the mutant mice have to excrete more urine and salt to maintain volume and electrolyte homeostasis. Since Ang II is a potent vasoconstrictor, its augmentation also leads to the development of hypertension and cardiac hypertrophy in VDR null mice, although the latter effect still needs more experimental verification. This is not unexpected, as hypertension and cardiac hypertrophy have been well documented in patients and animals with high renin and Ang II (37, 38). Thus, a new steady state of the renin-angiotensin system is established in VDR-null mice, in which the basal renin expression is higher but still responds appropriately to the same tubular salt load and volume stimuli as in the normal state. Based on these assessments, it is believed that the upregulation of renin expression is a primary defect in VDR-null mice.

That VDR-null mice maintain a high level of renin expression is, to our knowledge, a novel finding, but

the underlying physiological cause can be complicated. The observation that renin expression in VDR-null mice reacts properly to high salt load or dehydration indicates that the mechanism underlying the sustained renin elevation is independent of the pathways activated by tubular salt load or volume depletion. In fact, the involvement of cyclooxygenase-2 (COX-2), which may play an important role in macula densa-mediated renin release (39), in renin elevation in VDR-null mice is unlikely, since we observed the same low COX-2 protein level in the kidneys of both VDR-null and wild-type mice (data not shown). Since adult VDR-null mice develop hypocalcemia and secondary hyperparathyroidism, the upregulation of renin expression could be due to VDR inactivation per se, hypocalcemia, and/or high PTH. However, several lines of evidence from our study strongly suggest that vitamin D regulation of

Figure 8

1,25(OH)₂D₃ suppresses renin gene transcription. **(a)** Expression of hVDR mRNA in stable As4.1 clones. P, parental As4.1 cells; #57, As4.1 clone 57 stably transfected with pcDNA-hVDR; V, As4.1 clone stably transfected with the empty vector pcDNA3.1. **(b)** Renin mRNA expression in As4.1 clone V (Vector) and #57 treated with ethanol (E) or different doses of 1,25(OH)₂D₃ as indicated. **(c)** As4.1-hVDR cells (clone #57) were transfected with pGL3-control, pGL-4.1kb, or pGL-117bp luciferase reporter plasmid and then treated with ethanol (black bars) or 10^{-8} M 1,25(OH)₂D₃ (white bars). Luciferase activity was determined 48 hours after transfection. Similar results were obtained in other stable clones (data not shown).



renin gene expression is direct and independent of the calcium status: (a) Prewaned VDR-null mice that have not yet developed hypocalcemia already show an elevated renin expression; (b) when the blood ionized calcium of adult VDR-null mice is normalized by the HCa-Lac diet, their renin expression and Ang II level are still elevated; (c) conversely, Gcm2-null mice, which are as hypocalcemic as VDR-null mice, do not manifest elevated renin expression; (d) in wild-type mice, reduction of $1,25(\text{OH})_2\text{D}_3$ biosynthesis also results in elevated renin expression, whereas injection of $1,25(\text{OH})_2\text{D}_3$ leads to reduced renin expression; and (e) $1,25(\text{OH})_2\text{D}_3$ directly suppresses renin gene transcription in As4.1 cells by a VDR-mediated mechanism. Therefore, our data provide very compelling evidence to establish that vitamin D is a potent negative endocrine regulator of renin expression in vivo.

Although we did not observe a stimulation of renin expression in As4.1 cells either treated with PTH, or transfected with the PTH/PTHrP receptor and then treated with PTH, we cannot, at this time, completely exclude the possibility that, in vivo, secondary hyperparathyroidism may also contribute to the renin upregulation in VDR-null mice. This is because the serum PTH level in the normocalcemic preweaned or HCa-Lac diet-treated VDR-null mice is still significantly higher than that of the wild-type mice (even though it is much lower than that of the untreated adult VDR-null mice). Previous studies have shown that intravenous infusion of PTH increases plasma renin activity and renin release in humans and animals (33, 40), but the molecular mechanism whereby PTH regulates renin expression in vivo remains unknown. PTH may indirectly regulate renin expression in vivo.

$1,25(\text{OH})_2\text{D}_3$ exerts its actions by binding to the VDR. In most cases where $1,25(\text{OH})_2\text{D}_3$ acts as a positive regulator, the liganded VDR heterodimerizes with the RXR and binds to specific DNA sequences (VDRE) in the promoter of target genes to regulate gene expression. On the other hand, $1,25(\text{OH})_2\text{D}_3$ can also act as a negative regulator, but the mechanism of the negative regulation is more complicated and only partially understood. For instance, inhibition of other transcriptional complexes by VDR-RXR heterodimer or VDR homodimer (41, 42), interaction of VDR-RXR heterodimer with corepressors (43), and binding of VDR to a negative VDRE (44) have been reported for the VDR-mediated transcriptional repression. We postulate that $1,25(\text{OH})_2\text{D}_3$ suppresses renin gene expression through a *cis*-DNA element(s) in the renin gene promoter. Analysis of the renin gene promoter is underway to elucidate the molecular mechanism.

In summary, we have demonstrated that vitamin D functions as a novel negative endocrine regulator of the renin-angiotensin system in animals. Our data indicate that maintaining a normal level of serum $1,25(\text{OH})_2\text{D}_3$ is important not only for calcium homeostasis, but also for the homeostasis of electrolytes, volume, and blood pressure.

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